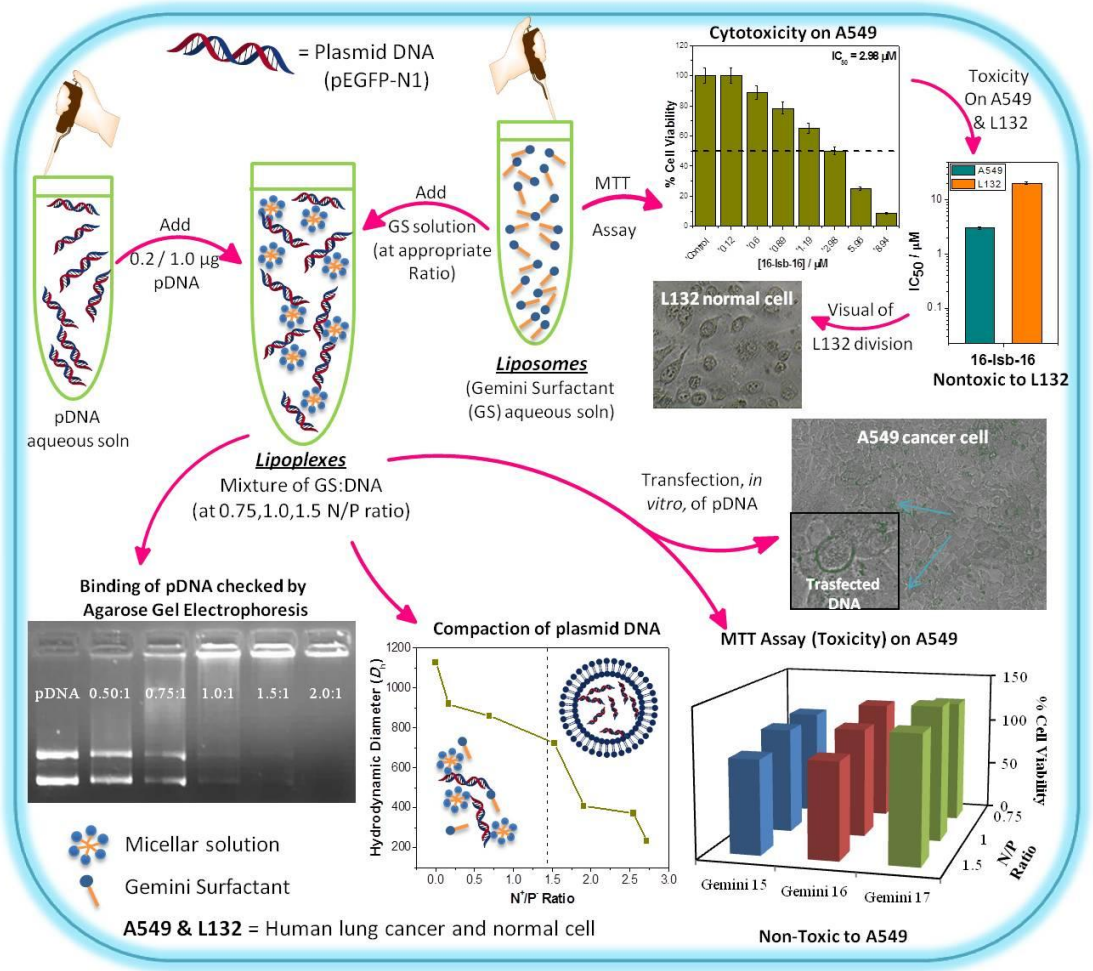
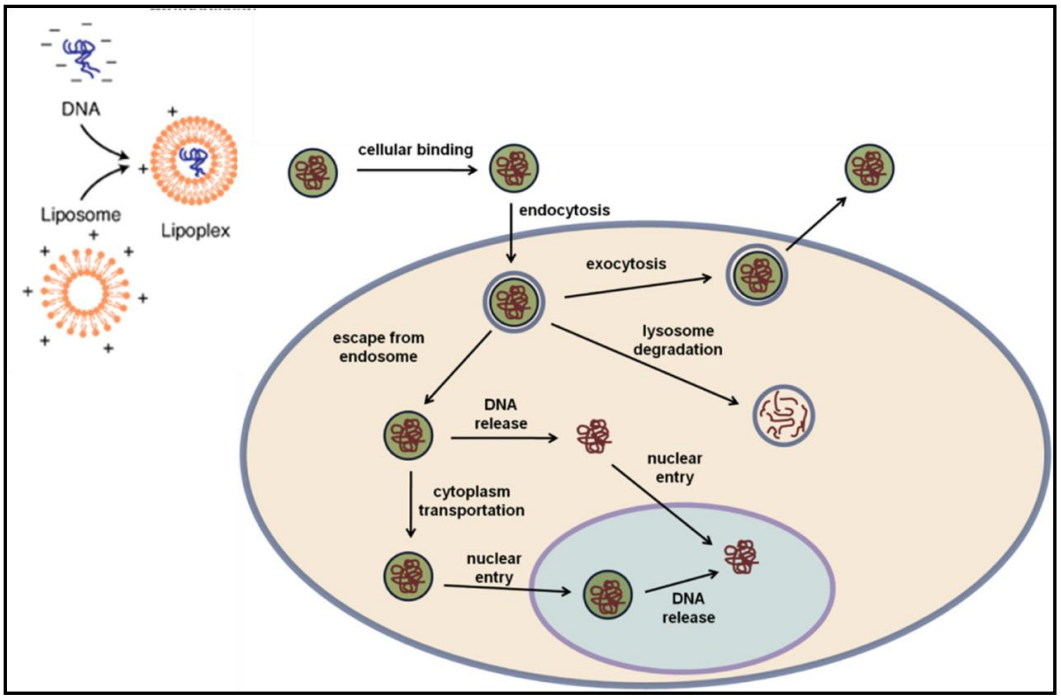
Chapter 6

Transfection, *in vitro*, of pEGFP-N1 by Cationic Gemini Surfactants on Human Lung Cancer Cell (A549)



6.1. Introduction

In modern medicine field, most challenging and exploring problem is to exploit a proper viral or non-viral vector for gene therapy [1]. Viral vectors are very much efficient to deliver the required genetic material to the nucleus of the cell or membrane in animal models but still prohibited on human clinical trials due to their demerits (antigenicity, toxicity, low drug volume and virus produced in a large scale) [2]. In today's world, non-viral vectors are in demand because of ease of their synthesis, low immune response, safety factors and large-scale production [3]. However, these non-viral vectors are not as efficient as the viral ones. There is an interest in choosing proper non-viral vector *in vitro* from cationic lipids, lipopolymers, polymers, cationic gemini lipids, peptides, among others [4]. Since the first report by Felgner's group in 1987 on a cationic lipid (*N*-[1-(2, 3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)) based non-viral vector gene therapy *in vitro* [5], much attention has been received from the medicinal world for similar material. The structure of cationic lipids has further been modified to increase the efficiency of the non-viral vector [6]. Recently, cationic gemini surfactants as a vector (*carrier*) in gene transfection studies, *in vitro*, have been the focus of numerous researches due to their better bio-physiochemical properties [2*a*, 4, 7]. The properties include their ability to bind / condense DNA (it is generally called lipoplexes (complexes formed between surfactant / amphiphile and DNA at appropriate ratio)) and low cytotoxicity of lipoplexes towards the human cell-line [8]. Due to above properties, these vectors have good potential for better gene transfer. Mainly, lipoplexes (See Scheme 1) are the moiety which readily taken up by the cells through endocytosis phenomenon (cellular uptake, endosomal escape and nuclear entry) [9].



Scheme 1. Schematic representation of lipoplexes (aqueous mixture of surfactant and DNA) mediated transfection through endocytosis [9].

Generally, toxicity of lipoplex towards the cell is the main preliminary factor for the study. Recently, i*n vitro* studies, it has been reported that ester/bio-species containing lipid (surfactant) can enzymatically hydrolyzed to non-toxic molecules after transfecting the DNA and can safely removed from the living organisms [10]. Therefore, it is worthwhile to check transfection, *in vitro*, efficiency of presently synthesized biocompatible as well as polymethylene (for comparison) spacer based geminis (for chemical structure see Chapter 2) towards human cell lines.

Why Human Lung cancer cell?

It is well know that cancer is one of the most extremely heterogeneous desease for the cause of more than 25% human deaths [11]. Among all types of cancer diagnosed, lung cancer has been estimated as the most common cancer for several decades and for the human deaths too [12]. The lung cancer in human body mainly occurs due to tobacco (mainly smoking) and air pollution with a time lag of

approximately 20-30 years [12*d*]. Various therapies (chemotherapy, endocrine therapy and targeted agents / combinations of these therapies) and surgery (at the last stage) have been employed to treat the lung cancer. However, there are limited studies for treating the lung cancer cell by transfection of DNA, *in vitro and vivo* [2*a*, 4, 7].

In the present Chapter, studies on complexation and compaction of geminis with plasmid DNA (pEGFP-N1) using agarose gel electrophoresis and DLS measurements have been performed. Studies have also been performed on toxicity of lipoplexes (mixture of gemini and pDNA at desired N/P molar ratio) towards human lung cancer cells (A549) by using MTT assay. After binding and toxicity study, desired lipoplexes have been used for transfection (*in vitro*) study over to A549. In endocytosis phenomenon (mainly after cellular uptake), whether gemini surfactant remains toxic towards cell or not, MTT assay of all gemini surfactants towards A549 as well as on human lung normal cell (L132) has also been performed.

6.2. Materials and Methods

6.2.1. Materials

Escherichia Coli DH5α strain, Tris base, Acetic acid, EDTA, Agarose powder, Ethidium bromide, Chloroform (99.5%, Spectrochem), Dulbacco's Modified Eagle's medium (DMEM, Sigma-Aldrich), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) dye, 10% FBS, DMSO (99.5%, Spectrochem) were used as received. Plasmid DNA (pEGFP-N1), human lung cell lines (adenocarcinoma (cancer), A549; normal, L132) are purchased from National Centre for Cell Science

(NCC), Pune, India. Sterile de-ionized double distilled water (0.5-1.5 $\mu S \cdot cm^{-1}$) was used throughout.

6.2.2. Methods

6.2.2.1. Plasmid DNA (pEGFP-N1) Amplification

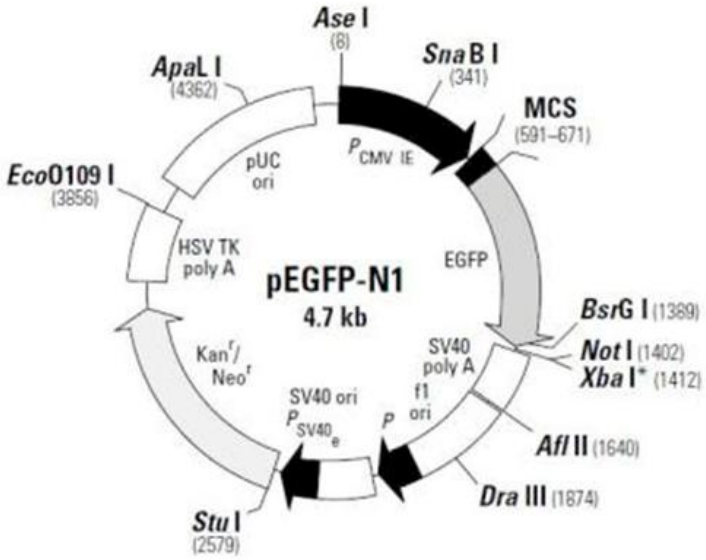


Figure 1. Structure of enhanced green fluorescence (EGFP) protein contain plasmid DNA (pEGFP-N1) having anti-kanamycin gene.

The plasmid DNA (pEGFP-N1) of 4.7 kb size, encoding enhanced green fluorescence (EGFP) protein under CMV promoter, has been used for the study (Figure 1). The plasmid DNA was amplified in the *Escherichia coli* DH5α strain. pEGFP-N1 has anti-kanamycin gene which allows transformed *E. coli* culture to grow selectively in Leuria Bertani (LB) medium containing 30 µg/ml kanamycin antibiotic at 37°C with agitation. Amplified pEGFP-N1 was extracted and purified using alkaline lysis protocol [13]. Purity of the plasmid was checked by electrophoresis on 1.0% agarose gel and by measuring the optical density (OD) of the plasmid preparations using spectrophotometer. The plasmid preparations, showing the values

of OD_{260}/OD_{280} ratio between 1.8 to 2.1, are used for interaction studies. Concentration of DNA has been estimated spectrophotometrically by measuring the OD at 260 nm.

6.2.2.2. Sample Preparation of Liposomes

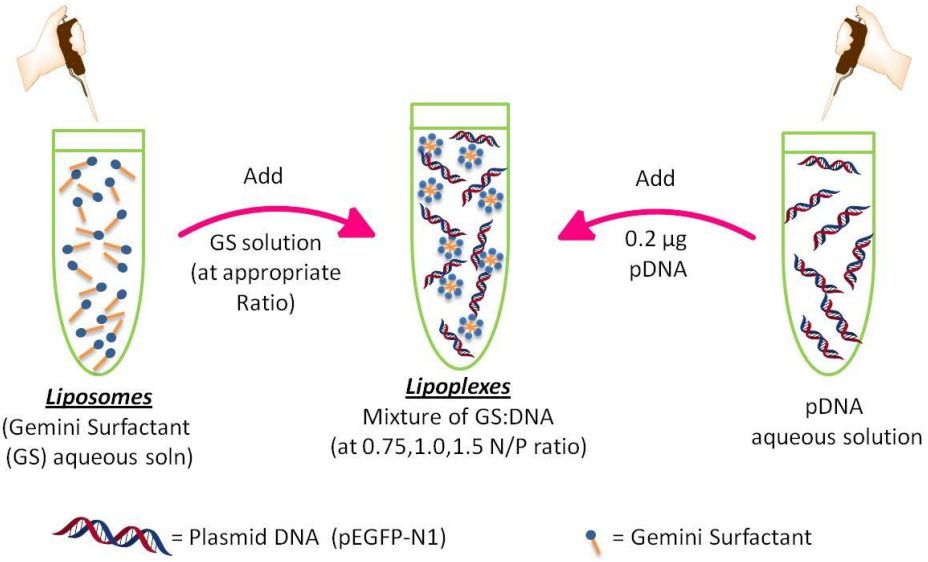
To prepare 0.5 mM solution for cytotoxicity and DNA binding, appropriate amount of individual gemini surfactant was weighed and dispensed in autoclaved Wheaton glass vials. Geminis were dissolved by adding chloroform in each Wheaton glass vial and dry thin film has been made by evaporating the chloroform under high vacuum (750 mmHg) for 6-7 hrs. After complete evaporation of chloroform, an appropriate volume of autoclaved distilled water was added in each vial containing surfactant dry films. The solutions were kept overnight for hydration at 4˚C. The hydrated surfactant layer was subjected to freeze-thaw cycles (ice-cold water to 60˚C) for 4-5 times with intermittent vortexing to ensure hydration. Subsequently, the solutions were sonicated in bath sonicator at 60˚C for 15 min. For cytotoxicity assay without DNA, stock solution of the gemini surfactant is prepared in Dulbacco's Modified Eagle's medium (DMEM, Sigma-Aldrich). All the suspensions were prepared and kept under sterile conditions.

6.2.2.3. *In Vitro* Cell Culture.

The cell lines (A549 and L132 cells) were pre-cultured in DMEM supplemented medium (containing 10% fetal bovine serum and 1% antibiotics) in 10 cm culture discs / 96 well culture plates. Cultured cells were incubated at 37˚C in humidified atmosphere containing 5% CO_2. DMEM medium was changed every third day and cell growth was monitored microscopically. Cells were regularly trypsinized (0.1% trypsin EDTA 0.02%, dextrose 0.05%).

6.2.2.4. Agarose Gel Electrophoresis

Lipoplexes are formed by mixing the gemini surfactant (**7-9**) and the plasmid DNA at different molar ratios (0.75, 1.00, 1.50, 2.00 and 3.00). To achieve the desired gemini/DNA molar ratio, 0.2 µg of DNA was mixed with appropriate volumes of 0.5mM gemini surfactant stock solution in separate micro-centrifuge tube followed by incubation at room temperature for 30 min (Scheme 2). The electrophoretic mobility of the gemini surfactant-DNA complexes, at different molar ratios, along with pure uncomplexed DNA was determined by gel electrophoresis using 1% agarose gel containing 2 µl of ethidium bromide (0.5 mg/ml) in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA). Electrophoresis was carried out at 100 V for ~45 min at room temperature.



Scheme 2. Schematic representation of formation of lipoplex (mixture of gemini surfactant and DNA) and liposome (aqueous solution of gemini surfactant).

DNA bands can be visualized by exposing the gels under UV transilluminator (Alliance 4.7 UVITEC) at room temperature. Digital images have been captured using the Alliance UVITEC camera. Adobe Photoshop software (Adobe Inc., San Jose, CA,

USA.) is used to assemble images into figures. No post-acquisition modifications are made to the original images.

6.2.2.5. Compaction Study

For compaction study, The DLS measurements have been performed. Detailed methodology related to DLS measurements has already been described in earlier chapters (see Chapters 3 and 4).

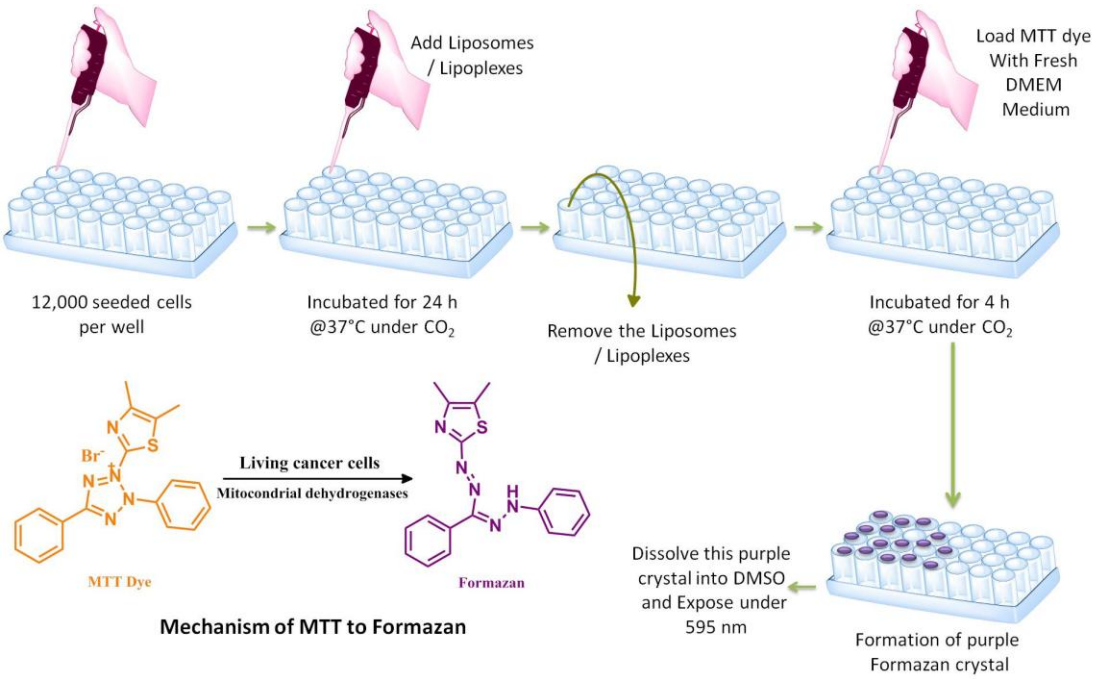
6.2.2.6. Cytotoxicity (MTT) Assay

The cytotoxicity assay using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] has been performed [14] with liposomes / lipoplexes to evaluate the toxicity on A549 (lung cancerous) and L132 (lung normal) cells. Both the cells were seeded separately on 96-well microplates (Nunc, Denmark) at the density of 12,000 cells per well and were allowed to grow for 24 h in DMEM medium containing 10% FBS. The cells were incubated at 37ºC in a 99% humidified atmosphere containing 5% CO_2. To check the toxicity, A549 and L132 cells were treated with a wide concentration range of liposomes (aqueous solution of gemini surfactant) and with gemini/DNA mixture at different molar ratios (0.75, 1.0 and 1.5). Prior to the A549 cell exposure, the gemini/DNA solutions were incubated for 30 min at room temperature to allow the formation of lipoplexes. Liposomes and lipoplexes both were added to culture media and incubated for 24 h. After incubation, culture media containing lipoplexes or liposomes were replaced with 200 μl of fresh DMEM media containing 10% FBS and 20 μl MTT dye solution (5 mg/ml in PBS) was added to the media in each well. After addition of MTT, the culture was further incubated for 4 h at 37°C to allow the formation of purple formazan crystals (indication of live cell). After 4 h incubation, the medium was removed very carefully and 200 μl of

DMSO was added in each well. The culture plate was covered and incubated at room temperature with gentle agitation for 10 min to dissolve the purple crystals. The absorbance was measured at 595 nm using a micro-plate reader. Percentage cell viability was calculated from absorbance reading using the following equation (Eq. 1),

$$\% \text{ Viability} = \frac{A_{\text{treated cell}} - A_{\text{Blank}}}{A_{\text{Untreated cell}} - A_{\text{Blank}}} \text{ X } 100 \qquad (1)$$

Experiments were performed in triplicates to minimize the error. In case of liposome (without DNA), cytotoxcity assay were calculated from % cell viability *vs* [surfactant] responsive curve to have IC_{50} value (which represents the concentration of each gemini surfactant that lower the MTT reduction by 50% compared to untreated control). 5-10 % error bar represents the uncertainty for each value.



Scheme 3. Schematic representation of MTT assay for cytotoxicity.

6.2.2.7. Transfection Procedure

To check the gene delivery efficiency of different gemini formulations, transfection experiments are performed on human lung cancer cell (A549). The procedure followed as per commercially available transfection reagent 'Lipofectamine 2000'. A549 were plated in 24-well plates as 50,000-60,000 cells per well in antibiotic free 10% FBS containing DMEM medium. Cells were grown for 24 h at 99% humidity, 37°C temperature and 5% CO_2 conditions. For all the experiments, desired lipoplex concentrations are formed by mixing appropriate molar ratio (where most binding and non-toxicity are observed) of 0.7 µg plasmid DNA and geminis in separate micro-centrifuge tube and followed by incubation at room temperature for 30 min. The lipid concentrations have been varied to obtain the required lipid/DNA (N/P) charge ratios. Before loading of lipoplex, old medium was removed from the wells followed by washing of cells with DMEM. Lipoplex in 200 ml media per well were added to the cells. The plates have been incubated for 6 h at ambient condition. Old medium was removed from the wells after 6 h of incubation. In both the cases, at the end of the incubation period, the medium was removed and the cells were washed with DMEM (500 ml of DMEM containing 10% FBS was added per well). Plates were, further, incubated for a period of 42 h before checking the level of reporter gene expression. GFP expression is examined by fluorescence microscopy (at 20x on Floid cell imaging station, life technologies).

6.3. Results and Discussion

6.3.1. Optimization of N^+/P^- Molar Ratio by Agarose Gel Electrophoresis

Molecules with opposite charge can easily bind each other through charged sites, which is a well-known phenomenon in interaction process. The same phenomenon has been performed through electrophorectic gel retardation assays to characterize the electrostatic interaction between plasmid DNA and aqueous solution of gemini surfactants (liposomes) at different N^+/P^- ratios (surfactant/DNA molar ratios) which retards the movement of plasmid DNA towards cathode.

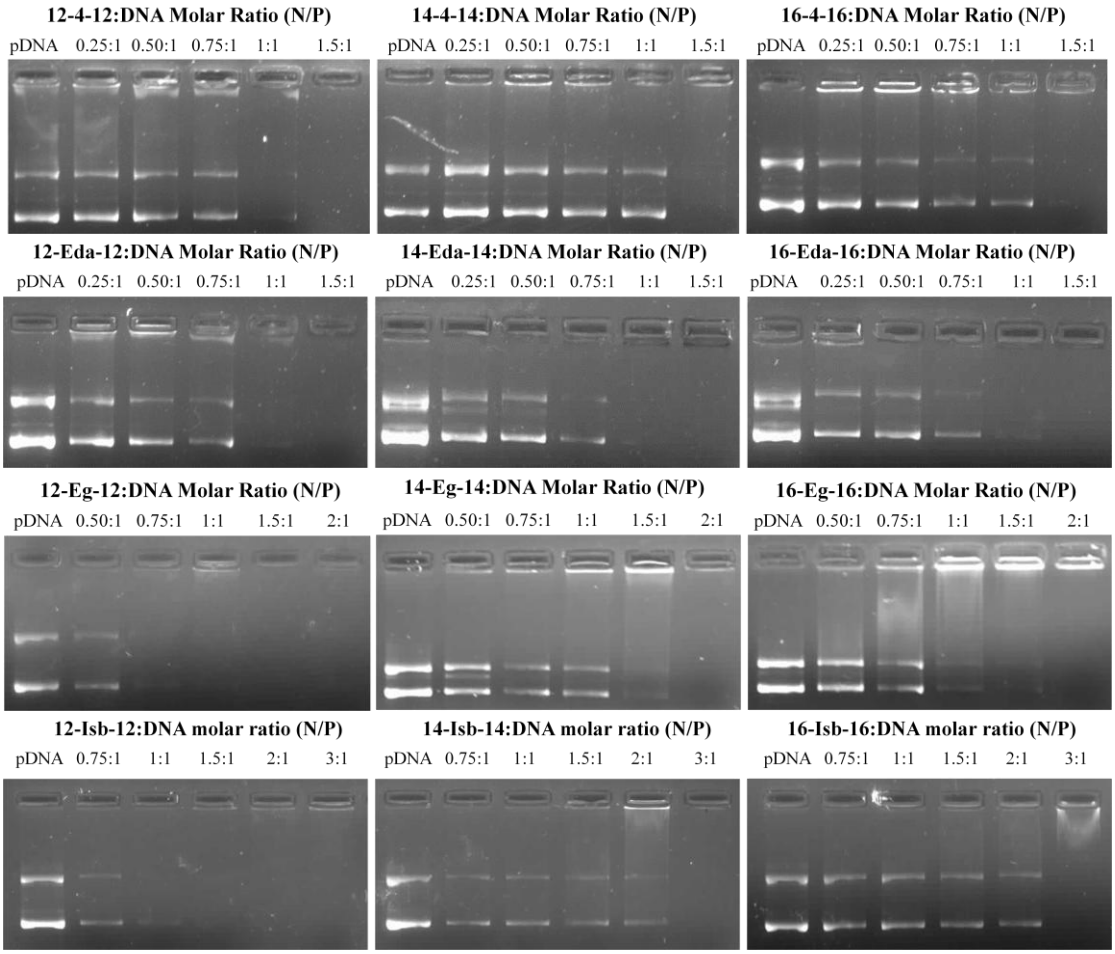
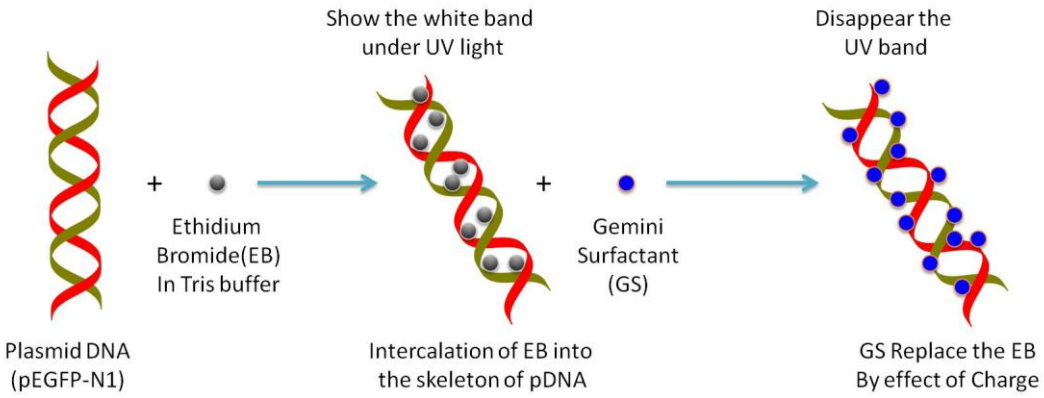


Figure 2. Agarose electrophoretic gel patterns for gemini surfactants associated DNA at different N^+/P^- molar ratio and run on 1.0% agarose gel. To minimize the error the experiments were carried out in triplicates. Top of the line indicated the surfactant and second line is for N^+/P^- charge ratio. 0.2 µg pDNA was used complexed with each surfactant at different N^+/P^- molar ratio and incubated for 30 min.



Scheme 4. Schematic representation of gemini:DNA binding mechanism in agarose gel electrophoresis.

Figure 2 shows the visible bands under UV light appeared due to intercalation of ethidium bromide (EB) in DNA. After addition of different N^+/P^- molar ratio of gemini:DNA, disappearance of UV visible bands have been observed due to the replacement of intercalated EB by gemini surfactants. Disappearance of band was showing ultimately the binding N^+/P^- ratio of DNA and geminis (Scheme 4). All the biocompatible and polymethylene spacer based cationic gemini surfactant formulations (liposomes) were able to bind 100% with negatively charged DNA at appropriate N^+/P^- molar ratio. This suggests that there is a complete neutralization of charged DNA via formation of stable lipoplex (complexation of plasmid DNA and liposomes) at an appropriate molar (N^+/P^-) ratio. On comparing gemini surfactant-DNA interactions, 12-Eg-12 interact with DNA at 0.75 N^+/P^- molar ratio which was found more prominent than the other geminis (Figure 3). 42% of the cationic gemini surfactants were able to impede the DNA from the well at 1.0 N^+/P^- molar ratio, while 33% of the cationic gemini surfactants were restrict the DNA in to the well at 1.5 N^+/P^- molar ratio. Rest of geminis (14-Isb-14 or 16-Isb-16) have bind with the DNA at 3.0 N^+/P^- molar ratio. It is noted that the cationic gemini surfactant has two head groups (positive charges), therefore, equal binding surfactant:DNA molar ratios of geminis would be ~0.375, ~0.5, ~0.75 and ~1.5 for 0.5, 1.0, 1.5 and 3.0 N^+/P^- ratio,

respectively. However, in case of amide spacer based geminis binding ratio was similar that of monomeric conventional surfactant (and even not influenced by the ***m***). The binding composition of 12-Eg-12 was even smaller than the conventional one. Therefore, the ester $(-(CH_2)_2\text{-}OCO\text{-}CH_2)_2$ based gemini surfactant with lower ***m*** can influenced the plasmid DNA binding affinity more than the other.

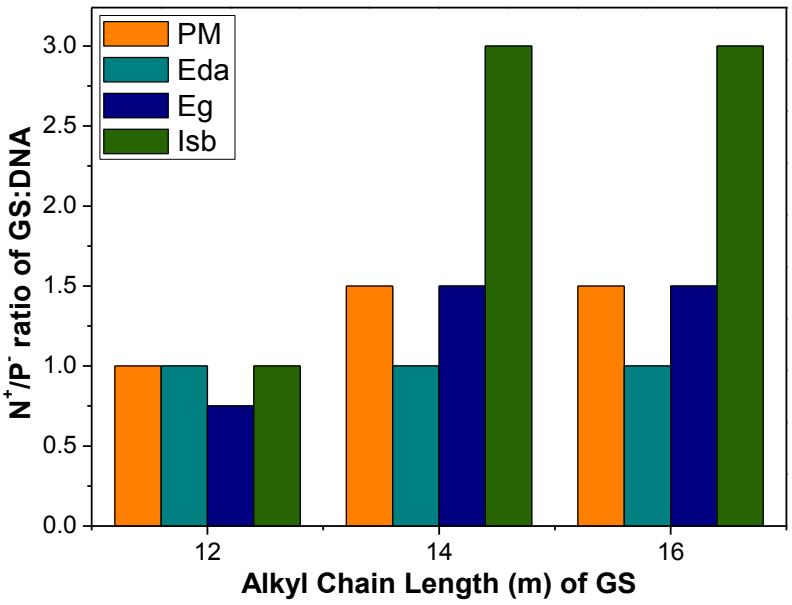


Figure 3. Representative plot of gemini surfactant:DNA binding molar (N^+/P^-) ratio *vs* alkyl chain length (***m***) of gemini surfactants (GS).

Data (Figure 3) showed that nature of the spacer can play a dominant role in binding affinity towards DNA. DNA binding capability was also found dependent on ***m*** only for ester or isosorbide based geminis. Therefore, replacement of ester / isosorbide to amide group in the spacer can enhance DNA binding. Flexibility of the spacer can also facilitate the morphological rearrangement in the system (i.e., micelle to vesicle transition) [15]. This major difference between the binding capabilities of surfactants may also be due to the intra-molecular and inter-molecular H-bonding. Thus, H-bonding tendency and flexibility of spacer (and lower hydrophobicity) can play a crucial role towards lipoplex formation.

Gemini surfactant and DNA binding can also be discussed in terms of physiochemical properties. Surfactant can bound with DNA below its cmc (this concentration is called crtical aggregation concentration, *cac*). This is because surfactant monomers can form loose micelle like structures near the binding site of DNA (above their *cac*). Due to strong attraction between the oppositely charged moieties, the effective concentration of surfactant (*cac*) around DNA would be higher than that in the bulk solution [16]. Few authors have discussed *cac* as pre-micellization concentration [8*a*, 16, 17]. The *cac* ([surfactant] directly used from where the complete neutralization of DNA was observed in the gel) of all the geminis (**5-7**, **10-12**, **15-17** or **20-22)** were found 1.56, 2.15, 1.99, 1.17, 2.15, 1.99, 1.56, 1.44, 1.33, 1.38, 3.83 and 3.58 μM, respectively. The *cac* values were found quite lower than the CMCs.

6.3.2. Compaction of Plasmid DNA

Plasmid DNA is well known for carrying any type of genetic code in to its skeleton for gene transfection but still one of the challenges for achieving the desired size of pDNA condensates [18]. In above section (6.3.1), gemini surfactant can easily neutralized the pDNA at appropriate N^+/P^- molar ratio which was quite necessary for docking into the cell membrane. However, study has not provided any compaction or condensation of pDNA (which facilitates to avoid the degradation of DNA).

In recent time, there were a few studies available to understand the protection / compaction of pDNA with the help of cationic gemini surfactants [19]. Similar mechanism may work in present case. Compaction of pDNA by 16-Isb-16 has been monitored by DLS measurements (Figure 4).

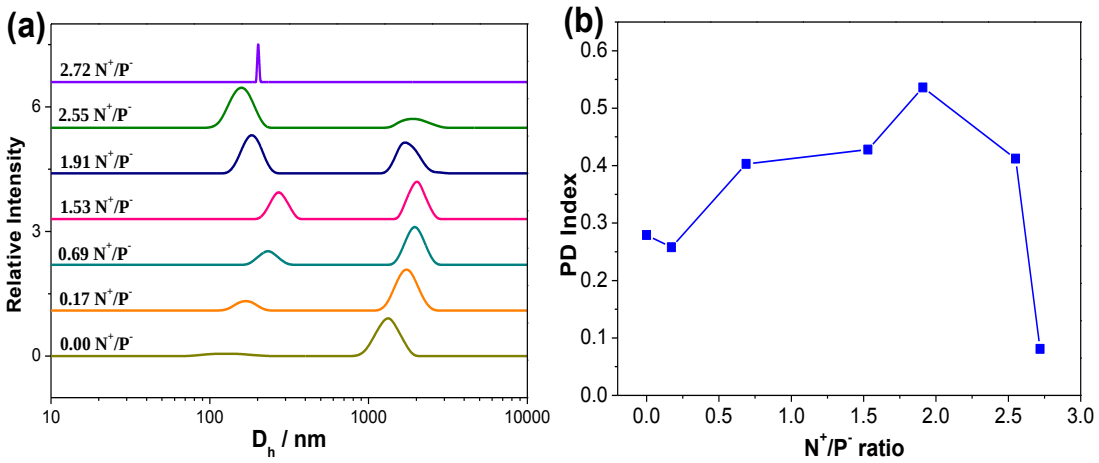
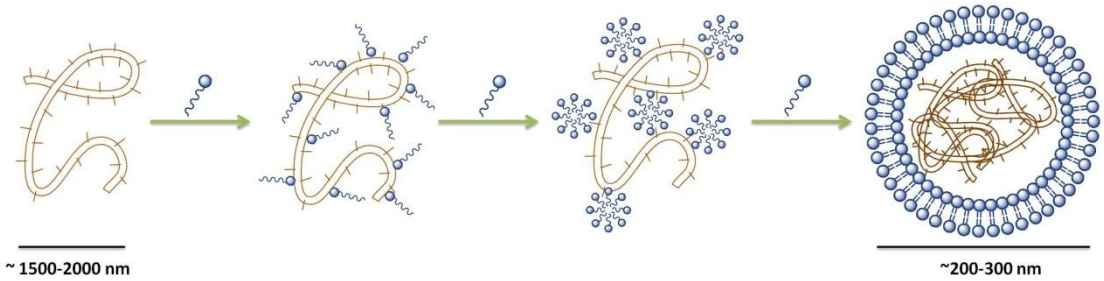


Figure 4. (*a*) Hydrodynamic diameter (D_h) at different N^+/P^- (gemini / DNA) ratio; (*b*) polydispersity (PD) index *vs* N^+/P^- ratio, of aqueous 16-Isb-16 system at 298 K.

In Figure 4*a*, hydrodynamic diameter (D_h) / size of pure plasmid DNA (pEGFP-N1) and complexes of DNA:gemini (16-Isb-16), at different molar ratio, are displayed. Pure DNA has size of ~1500-2000 nm in the form of monomodal size distribution (SD) which may be due to the helical and free form of DNA (see Figure 2). PDI value (0.279) is also supporting the presence of nearly mono disperse DNA. As [gemini] increase (up to 0.69 N^+/P^- molar ratio) two broad bimodal SD have been observed at ~200 and ~2000 nm, respectively (PDI value = 0.403). However, free DNA is dominating overall phase. The observance of lower size (in presence of gemini) indicates compaction of pDNA by forming a micelle like structure (below their cmcs called *cac*) at negatively charged DNA surface followed by formation of DNA entrapped vesicular structure (Scheme 5). On increasing the N^+/P^- molar ratio (> 1.53), more than 50% DNA get condensate. The PDI value ~0.54 is also confirming the observation. At 2.72 N^+/P^- ratio, broad bimodal SD has been converted in to narrow monomodal SD (size ~200 nm with lower PDI). This confirms the conversion into compact form.



Scheme 5. Schematic representation of compaction of plasmid DNA (pEGFP-N1) in presence of gemini surfactant.

Interestingly, whole pDNA compaction N^+/P^- ratio (2.72) was nearly similar to that of binding ratio (3.00) for 16-Isb-16 (Figure 3). In an earlier study [19*a*], it has been reported that [compaction] was much higher than the [binding]. However, in present case, these two concentrations are quite different. This may be due to its lower cmc which facilitates the binding at lower concentration with DNA.

6.3.3. Cytotoxicity due to Lipoplexes on A549

The toxicity of lipoplex is a basic requirement for the transfection through cell membrane. MTT based cytotoxicity assay (based on transfection protocols as reported for commercial available transfecting agent "Lipofectamine 2000") [20] was performed for all lipoplexes, with lower (0.2 µg) as well as higher (1.0 µg) DNA (fixed concentrations), on A549 at optimized gemini surfactant:DNA(N^+/P^-) molar ratios (0.75, 1.00 and 1.50 obtained through the gel retardation) and with fixed dose time (24h). The toxicity data are depicted in Figures 5 and 7. MTT assay gives percentage of viable cells that grew and divided after exposure. This is the proliferation of A549 cells due to non-toxic behavior of amphiphile / surfactant.

Figure 5 shows the cytotoxicity data at lower (0.2 µg) pDNA content. Data revealed that each lipoplex formulation showed very low toxicity towards A549 even

at high N^+/P^- molar ratio. Compared to all geminis, enhanced cell viability of 16-Eda-16 lipoplexes (at all N^+/P^- molar ratio) has been observed which represents the nontoxic nature of lipoplex (required for transfection). In general, when 80% pDNA neutralized by the gemini, the resulting lipoplex has been found non-toxic to the A549 (Figure 4). Hence, these non-toxic lipoplex can directly be used for the pDNA transfection. However, there has been no general trend found regarding the structure of the gemini (spacer or ***m***) and its effect on cytotoxicity.

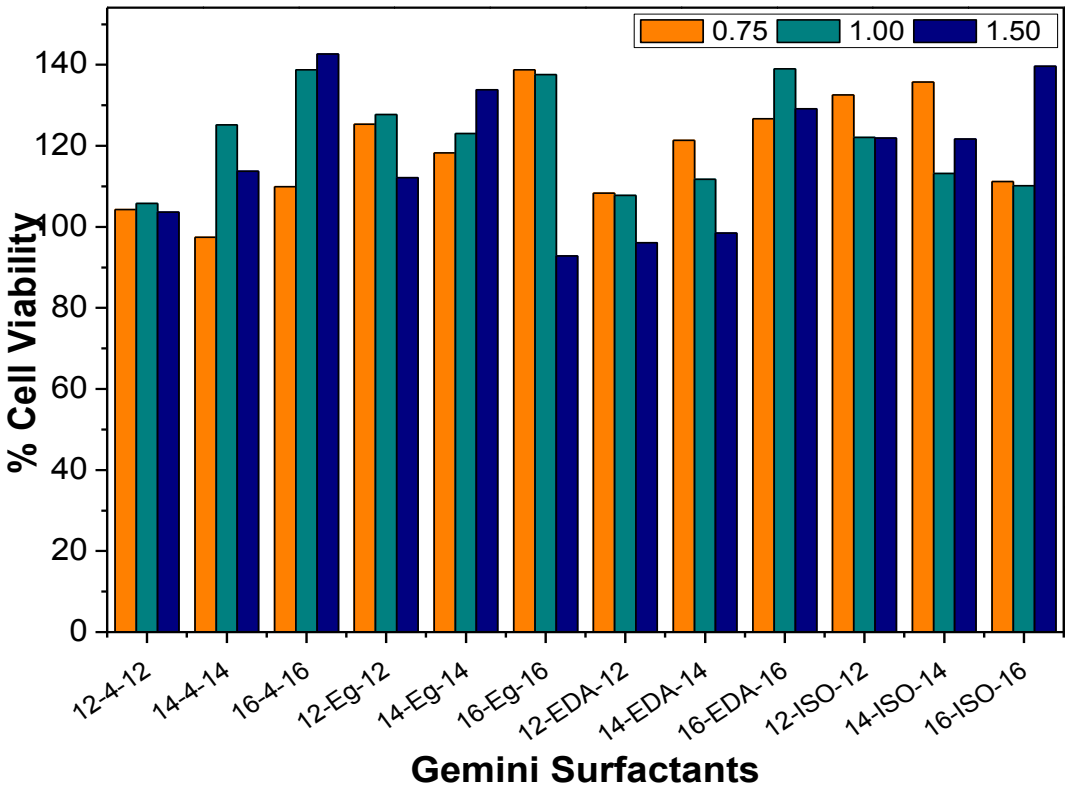


Figure 5. Cell viabilities of lipoplexes (surfactant:pDNA) over to A549 at different N^+/P^- molar ratio for all gemini surfactants at lower (0.2 µg) pDNA concentration.

To get further insight in to the loading of lipoplexes at different N^+/P^- ratio, electron microscopy has been used to visualize pDNA loaded A549 with and without lipoplxes (0.2 µg). The images (Figure 6) are showing uniform structure of cell membrane. Small globular structures have been observed on to the surface of A549 (zoomed image in Figure 6) with immediate loading of lipoplexes (12-Isb-12:pDNA

(0.2 µg)). These structures from the surface of A549 disappeared after a short time (~5 min.) indicating the proliferation of A549 cell by the lipoplex. Similar observations are also made with other lipoplex combination but data could not be captured (images) due to small life time.

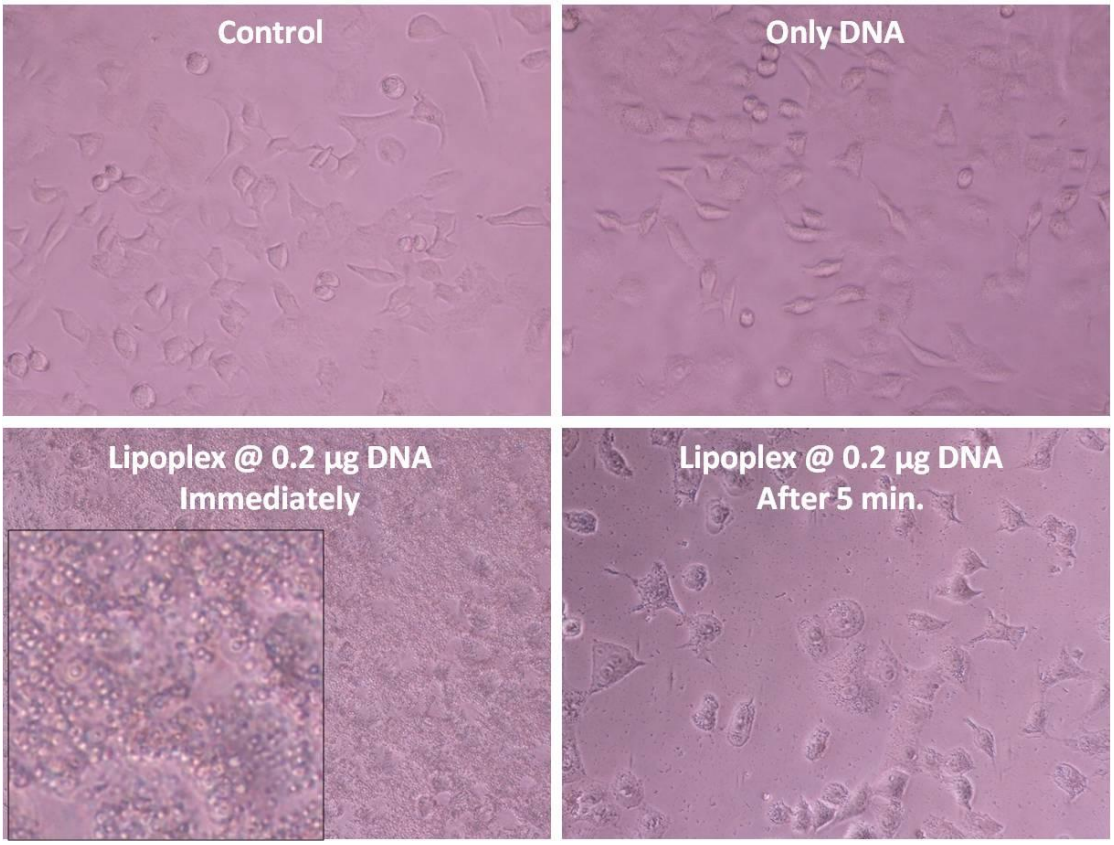


Figure 6. Electron microscopic images (~20x magnification) of treated / untreated A549 cells with lipoplexes @ 0.2 µg pDNA immediately and after 5 min of 12-Isb-12.

To check the effect of higher pDNA (1.0 µg) concentrated lipoplex towards A549, similar MTT assay studies have also been performed (Figure 7). Interestingly, 12-Eg-12 and 12-Isb-12 are found less toxic (more than 100% cell viabilities) at all N^+/P^- molar ratio (12-Eda-12 and 12-4-12 shows exactly opposite trend). Ester and isosorbide spacer based gemini surfactant (***m***=12) can provide even better environment for living cells at lower as well as higher [DNA]. With the increase of ***m***, cytotoxicities of polymethylene and amide spacer based geminis lowered with the

increasing of N^+/P^- ratio. However, ester and isosorbide spacer based geminis were found more toxic with increasing the ***m***. This is a clear indication about the relationship of toxicity with the nature of the spacer / ***m***.

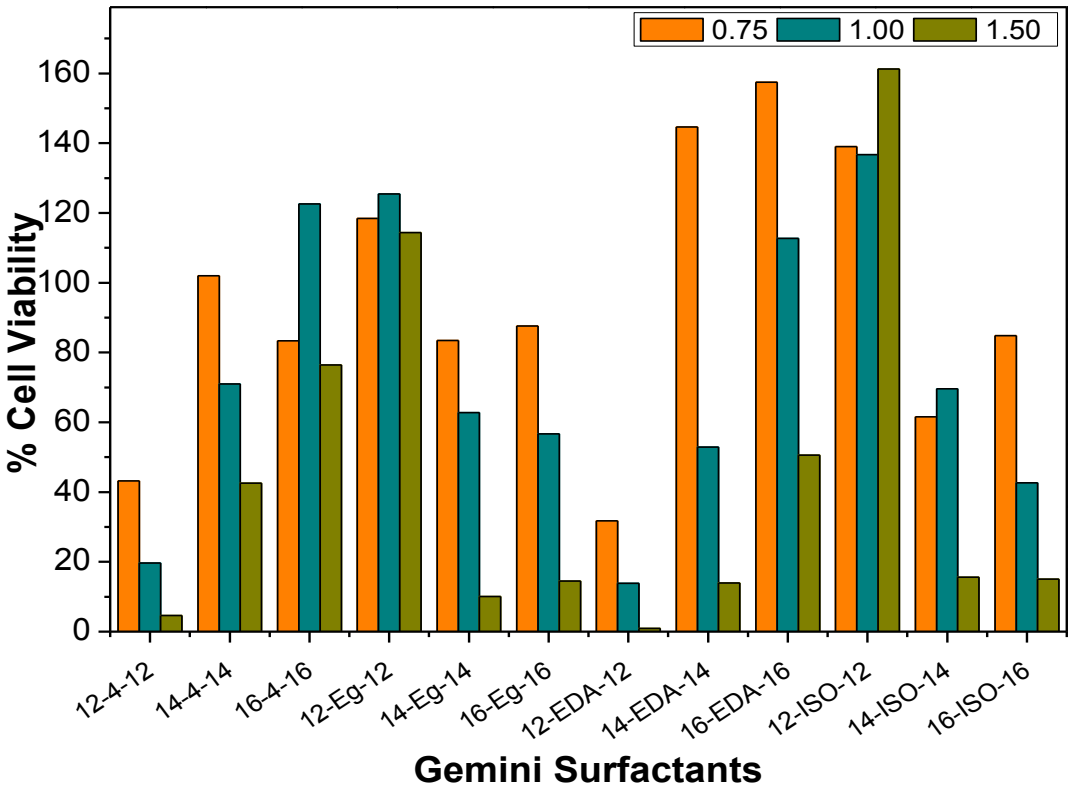
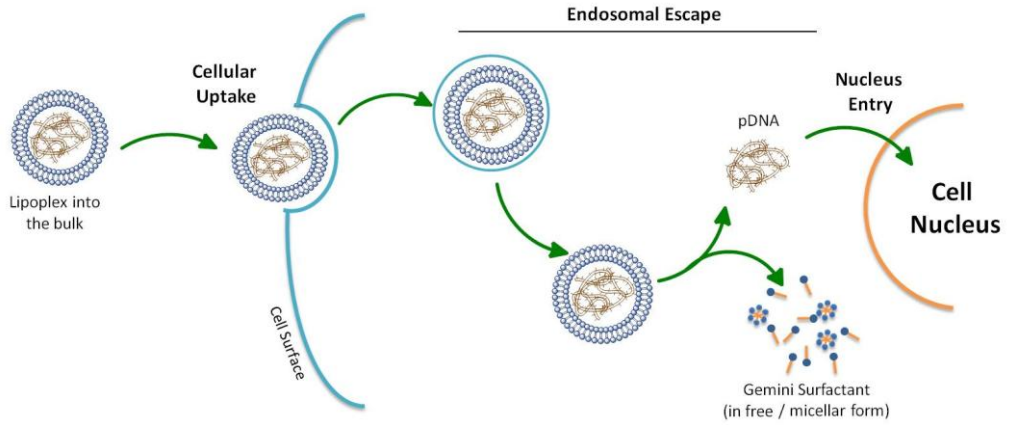


Figure 7. Cell viabilities of lipoplexes (surfactant:pDNA) over to A549 at different N^+/P^- molar ratio for all gemini surfactants at higher (1.0 µg) pDNA concentration.

The data clearly demonstrate that the concentration of pDNA also plays a significant role in the transfection process. The trend is more prominent with higher [pDNA]. The lower toxicity, of ester and isosorbide gemini based lipoplexes, may be due to the presence of more oxygen atoms in the spacers which facilitate effective binding (H-bonding would enhance the electrostatic attraction) with the surface of A549 [16]. The gemini surfactant toxicity behavior has also been checked without pDNA to confirm its role outside / inside the cell. The probable role of gemini / lipoplex in the interaction with cell can be represented by Scheme 5.



Scheme 5. Schematic representation of release of geminis surfactant after cellular uptake and endosomal escape of pDNA.

6.3.4. Cytotoxicity due to Liposmoes on A549 and L132

Lipoplex can enter and release the pDNA in to the cell (A549) through cellular uptake and endosomal escape. After release of pDNA, gemini may remain in monomeric / micellar form within the cytoplasm (Scheme 5). For trasfection, the toxicity of geminis in pure form is need to be known. Therefore, cell viabilities of all liposomes (aqueous solution of gemini surfactants) have been checked with both A549 and L132 using same MTT assay.

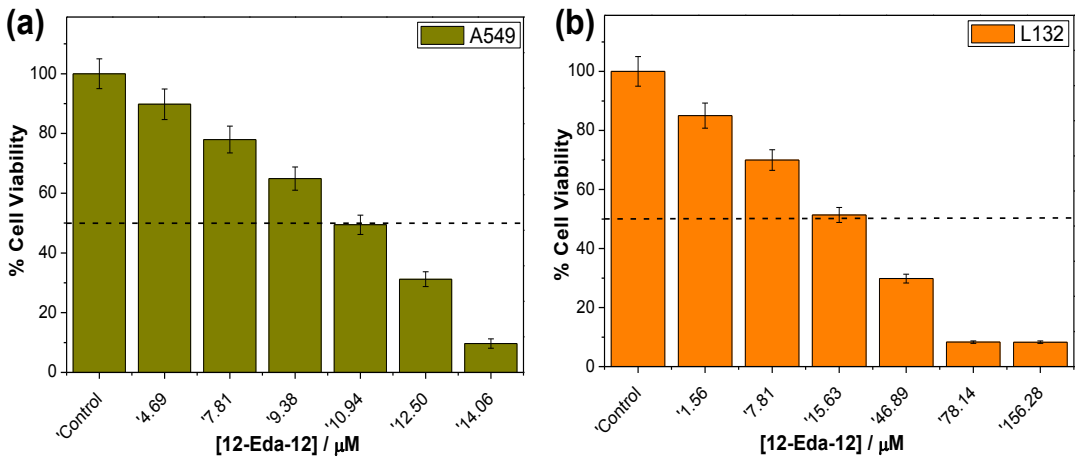


Figure 8. Representative % cell viability with ±5% standard deviation *vs* [12-Eda-12] for determination of IC_{50} values (indicating by dotted line) on (*a*) A549 and (*b*) L132.

Figure 8 shows that cell viabilities of both the cells are dose dependent (after 24 h). A wide concentration range of liposomes has been used to pin point the optimum range of cell viabilities (between two doses). This narrow range has been used for further MTT assay studies. The data has been used to obtain IC_{50} value (which represents the concentration of aqueous gemini surfactant solution that lower the MTT reduction by 50% compared to untreated control, Figure 8).

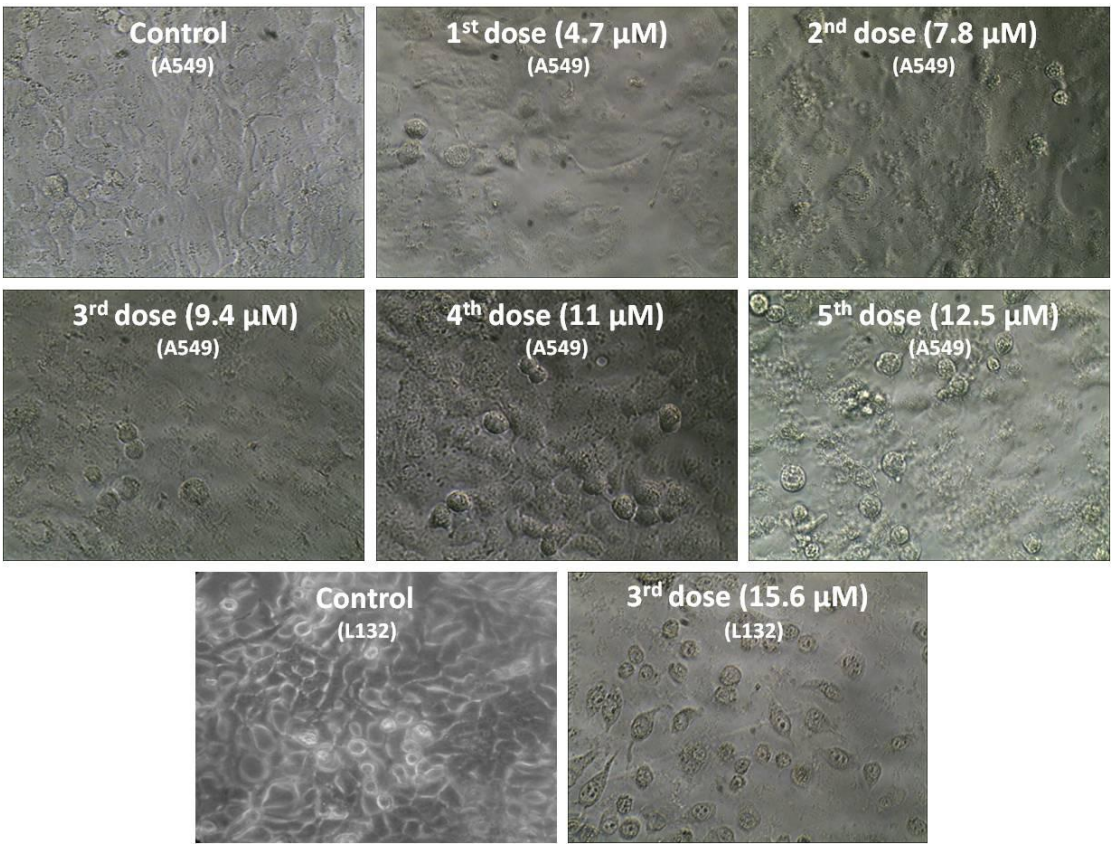


Figure 9. Electron microscopic images (~20x magnification) of A549 (cancer cell) and L132 (Normal cell) with increasing dose of aqueous 12-Eda-12 solution.

Electron microscopic study with selected samples (12-Eda-12 on A549 and L132) has been performed to get further insight (Figure 9). Perusal of the data hints that more small spherical structures (which were found similar to those of lipoplex mediated A549, Figure 6) are appeared as the concentration of liposome increases. Interestingly, more than 70-80% of A549 cells were manifesting by 12-Eda-12, while

at the similar concentration (15.6 µM), normal cells (L132) were still grew and divided into new cells (Figure 9).

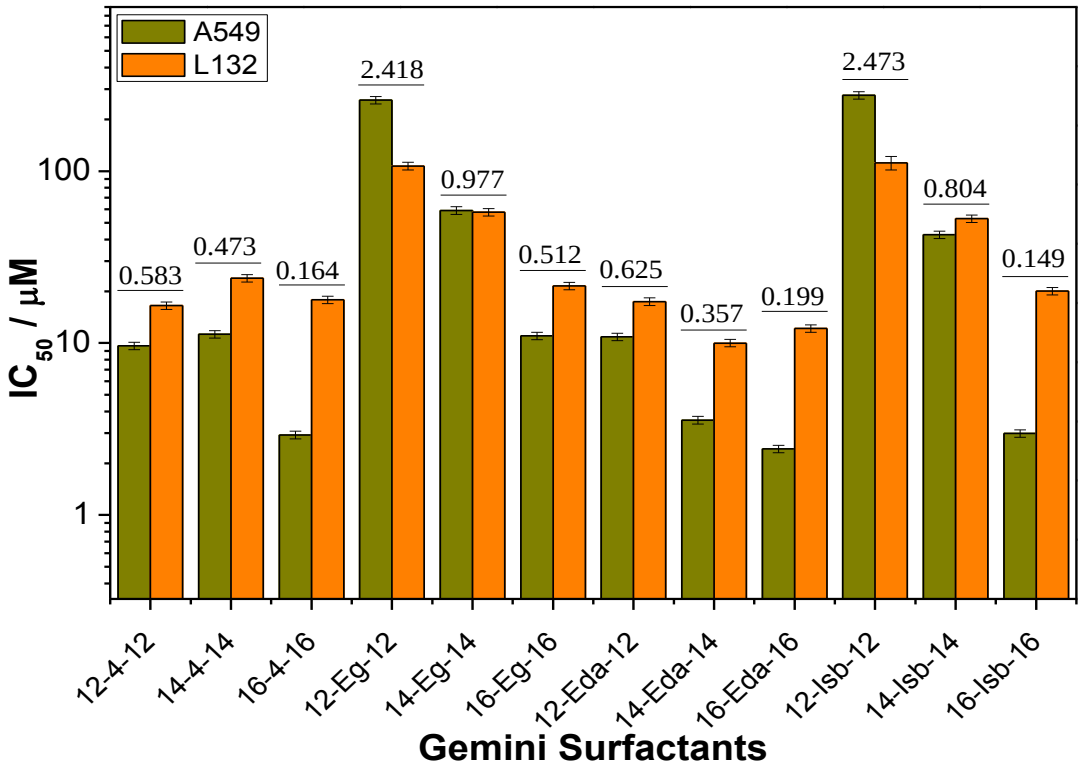


Figure 10. Plot of IC_{50} (µM) *vs* individual cationic gemini surfactants on human lung cancerous (A549) and normal cells (L132), respectively. Value over the histogram shows the ratio of IC_{50} values of gemini surfactants over to A549 and L132 (A/L).

The IC_{50} values of all liposomes on both cells are displayed in Figure 10. The low IC_{50} value indicates high toxicity of gemini towards cancer cells. Most of the geminis are showing high toxicity toward A549 but have no adverse effect on L132 at the same concentration (except 12-Eg-12 and 12-Isb-12). An opposite trend with 12-Eg-12 and 12-Isb-12 may be due to two factors: (a) preferential adsorption at the A549 - bulk solution interface; (b) cancerous cell has different size / shape and surface charge in comparison with the normal cell [21].

Similar information can also be drawn from the ratio of IC_{50} values of A549 and L132 (A/L in Figure 10) where the ratio is lower than 1.00 (viable for living

organism) in all cases. However, 12-Eg-12 or 12-Isb-12 has higher ratio (2.418 and 2.473) where overall concentration is 10 times higher than the other geminis with respect to A549.

It has also been observed that toxicity increases as the ***m*** of gemini increases for both the cell. Biocompatible spacer based gemini surfactants (with ***m*** = 12 and 14) are having higher IC_{50} values compared with polymethylene spacer based gemini surfactants. No such difference has been found in geminis with ***m*** = 16. If we consider a toxicity effect than 16-Isb-16, 16-4-16 and 16-Eda-16 were competing for better anti-cancer activity towards A549. Within them, 16-Isb-16 or 16-Eda-16 works even better for toxicity on A549 with minimal effect on L132 cells (due to biocompatibility). However, a cell death mechanism through liposomes has not been studied in detail. Surface porosity / breakage of the surface of the cell, by neutralizing the charge, have a role to play.

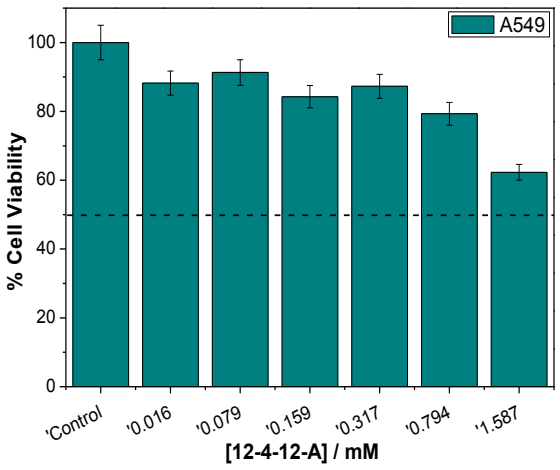


Figure 11. Plot of % Cell viability (with $\pm$ 5% standard deviation) *vs* concentration (µM) of 12-4-12-A for the determination of IC_{50} (dotted line at 50% cell viabilities indicates the IC_{50}) on human lung cancer cell (A549).

Cytotoxicity assay of phosphate head group based anionic gemini surfactant (12-4-12-A, for chemical structure see Chapter 2) has also been checked on A549.

Figure 11 shows that more than 65 % A549 cells remain viable even after exposure to 1.587 mM. On comparing toxicity results of cationic geminis (~0.2 mM) with 12-4-12-A (1.58 mM), 12-4-12-A has no adverse effect on A549 even at eight times higher concentration. Probably, the non-toxic effect of 12-4-12-A may be due to the same negative charge of the surfactant and cell. Therefore, charge (mainly positive) of the surfactant can play a crucial role for said manifestion of the cancer cells. From the cytotoxicity data (with and without DNA), one can say that toxicity concentration of liposomes (pure form of gemini) is much higher than the lipoplexes. Hence, after endosomal escape, gemini surfactant remain non-toxic towards the cell (throughout the tranfection study).

6.3.5. Transfection Study, *in vitro*

For transfection experiments on A549, gemini surfactant optimized conditions (complete neutralization of pDNA and formation of non-toxic lipoplex (at 0.2 µg pDNA)) have been directly used (see Figures 3 and 5). In a detailed study, it has been reported that maximum 4 h exposure is sufficient to achieve transfection of DNA towards A549 [7*i*]. Sample (A549 cells with GS:pDNA mixtures) incubated for 4 h and then lipoplexes are replaced by a fresh DMEM medium. Transfection efficiency has been examined by capturing visible and fluorescence microscopic images (Figures 12 and 13).

Light microscopy analysis of transfected cell shows the level of GFP protein expression. From Figures 12 and 13, one can see that 16-***s***-16 and 12-***s***-12 have higher transfection efficiency (except 12-Eda-12).

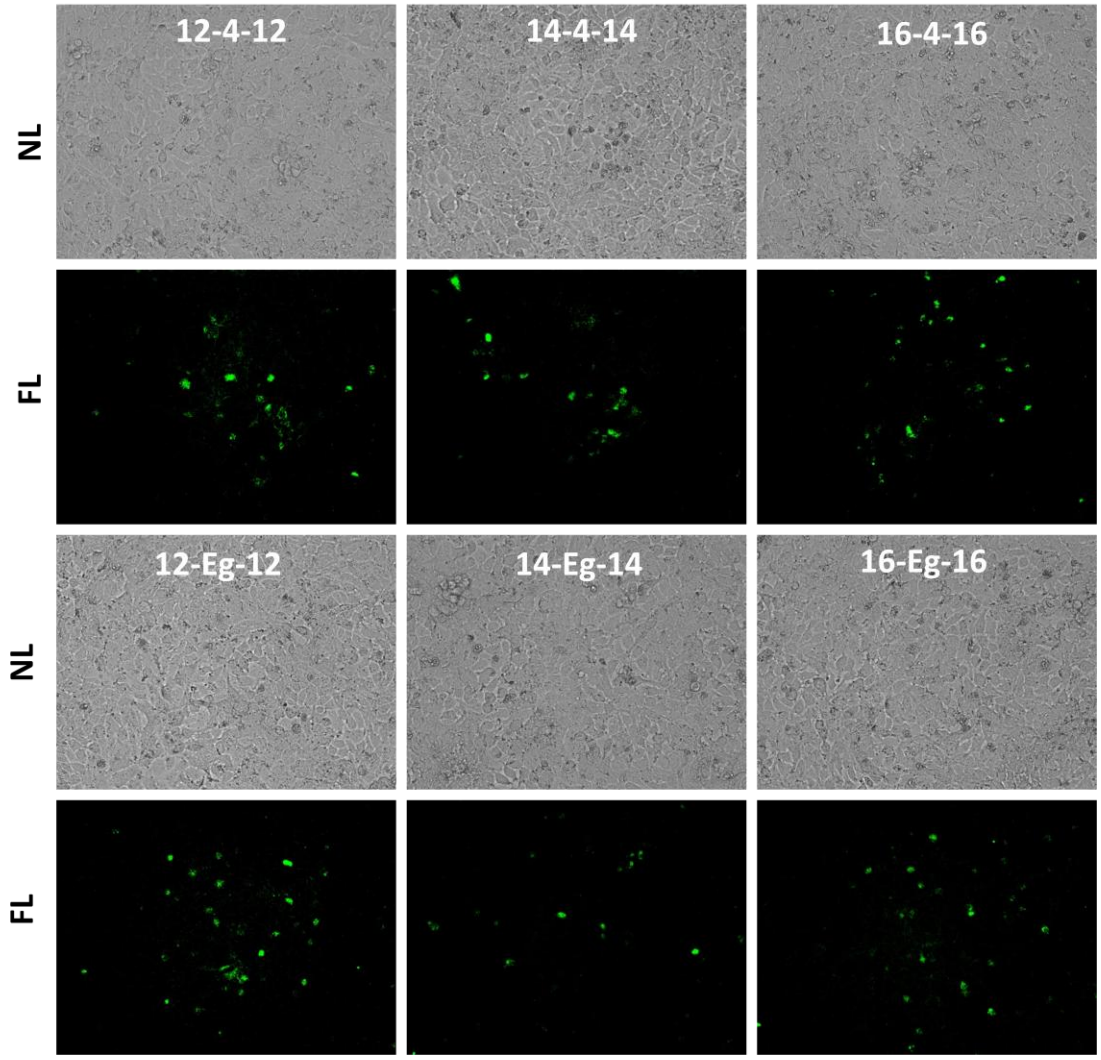


Figure 12. Human lung cancer cells (A549) transfected, *in vitro*, with polymethylene and ester based gemini surfactants with ***m*** = 12, 14 and 16, respectively. NL and FL means normal (visible) and fluorescence light.

Transfection efficiency of amide spacer based geminis follows the order: ***m*** = 12 > ***m*** = 14 > ***m*** = 16. Similar trend has not been followed with other geminis. Polymethylene, isosorbide and ester spacer based geminis show even better efficiency. The difference may be due to two factors: (a) isosorbide and ester based gemini molecules may have more hydration [22]; (b) amide spacer based geminis may have higher intra and inter-molecular H-bonding [10]. From comparison of transfection data (Figures 12 and 13), one can say that 12-4-12 and 16-Eg-16 have

been found better transfecting agent with respect to all three mechanism (cellular uptake, endosomal escape and nucleus entry).

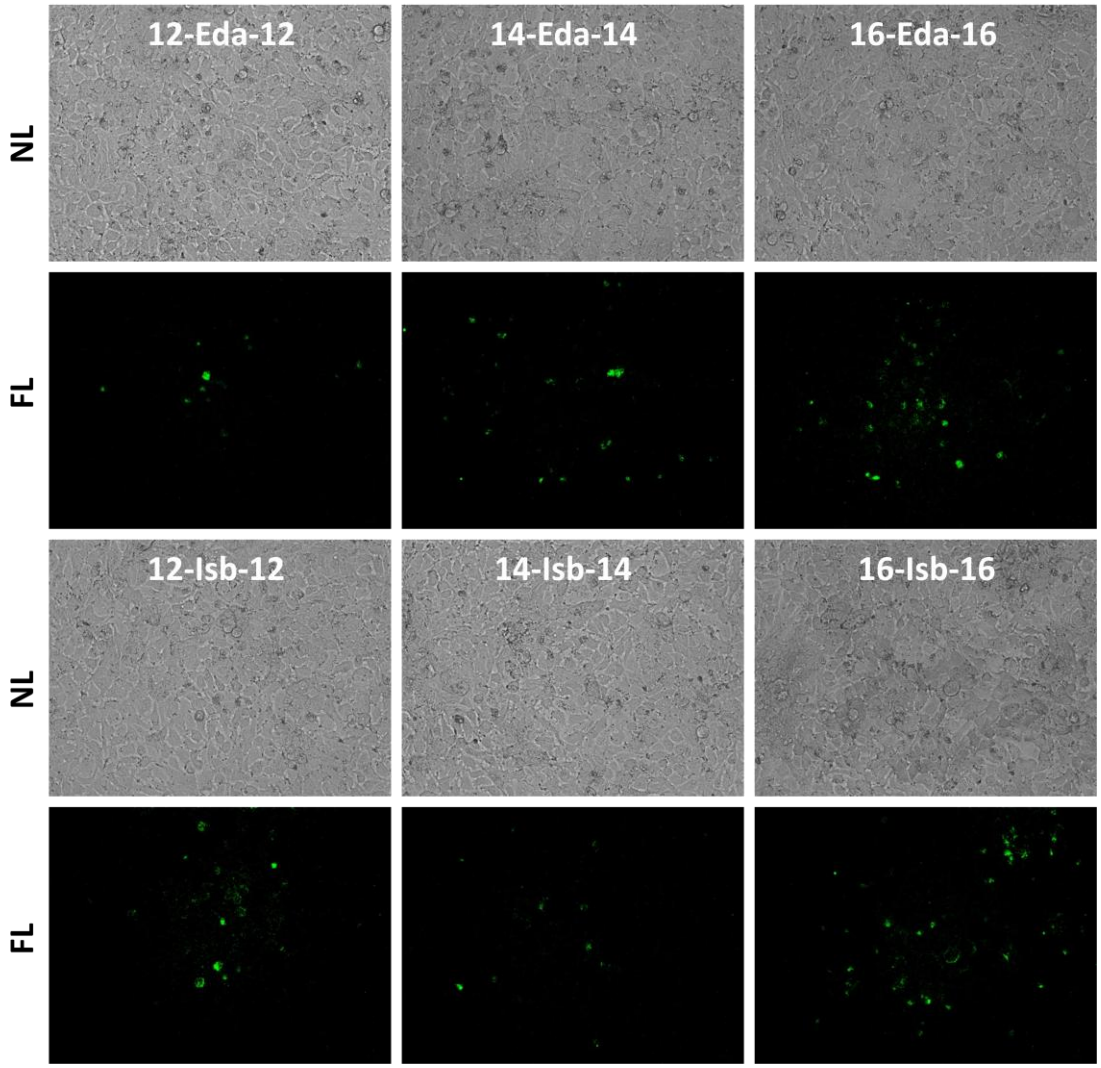
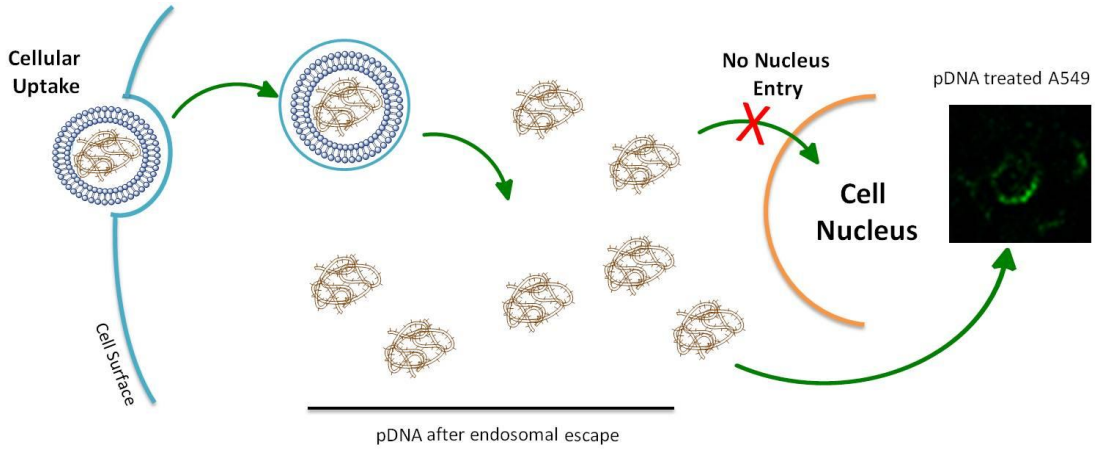


Figure 13. Human lung cancer cells (A549) transfected, *in vitro*, with amide and isosorbide based gemini surfactants with ***m*** = 12, 14 and 16, respectively. NL and FL means normal (visible) and fluorescence light.

In 12 series geminis (except 12-Eda-12), a few A549 cells are found only cellular uptake and endosomal escape (see Scheme 6). However, they failed to enter in to the nucleus of the cell. This may be due to three reasons: (a) time lapses (required more time); (b) pH difference or (c) destabilization of the endosomal / plasma membrane after lipoplex entry to the cell [10, 23]. However, most of the researchers are using helping lipids (e.g., DOPE, DOTAP, etc.) for avoiding such

endodomal escape and nucleus entry problem [24]. Here, most of the pDNA transfected / enter in to the nucleus of the cell with the help of single geminis including 12 series.



Scheme 6. Schematic representation of pDNA remains in cytoplasm.

6.4. Conclusion

All biocompatible and polymethylene spacer based gemini surfactants (more or less) have been found as a potential non-viral vector to deliver nucleic acid as plasmid DNA (pEGFP-N1) into the human lung cancer cell (A549) through various routes (e.g., cellular uptake, endosomal escape and nucleus entry, See Scheme 5). Effective binding / complete neutralization of 0.2 µg pDNA, compaction of pDNA and lower cytotoxicity of lipoplexes (content fixed 0.2 µg pDNA) are the possible factors helping in transfection.. However, cell viabilities (A549) of higher pDNA content lipoplexes (1.0 µg) have been dependent on nature of the spacer.

Cells were found even alive after cellular uptake and endosomal escape due to the lower toxicity of liposomes (aqueous solution of gemini surfactant). The toxicity of liposome without DNA has been observed much higher than the lipoplexes (0.2 and 1.0 µg). However, toxicity of liposomes has no adverse effect on L132 (human lung normal cells). Surface charge can also play a role in toxicity of the cell.

Compared to all geminis, ester and polymethylene spacer based geminis (***m*** = 12 and 16) have shown a better transfection efficiency (*in vitro*). 12-Eg-12 has better efficiency (due to its higher binding affinity towards pDNA) and lower toxicity towards A549. Transfection has been achieved with a single gemini surfactant which may be cost effective step towards the future application (*in vivo*).

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